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INCONGRUENCE BETWEEN CHLOROPLAST AND SPECIES PHYLOGENIES IN *EUCALYPTUS* SUBGENUS *MONOCALYPTUS* (MYRTACEAE)[1]

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Seventy-eight polymorphic cpDNA (chloroplast DNA) characters were found in 13 closely related taxa from *Eucalyptus* series *Amygdalinae* (subgenus *Monocalyptus*) and seven potential outgroup taxa. The strict consensus of six cladograms generated from cpDNA data confirmed monophyly of *Monocalyptus*. However, cpDNA phylogeny within *Monocalyptus* was incongruent with taxonomic classification, being more related to geography, even when accessions were from divergent series. *Monocalyptus* cpDNA formed two major clades. On the island of Tasmania cpDNA was restricted to a single clade, exhibited very little variation, and was phylogenetically related to cpDNA found in central and western Victoria. In contrast, cpDNA of mainland monocalypt taxa was more variable, even within the *Amygdalinae*. Four out of six Tasmanian *Amygdalinae* species were polymorphic. The difference between cpDNA of replicates was often greater than differences between species from different series. The low level of cpDNA variation and extensive morphological intergradation between the Tasmanian endemics suggest recent speciation. However, the transfer of cpDNA through hybridization between lineages is the most likely explanation for the observed sharing of cpDNA across series. This study highlights that the geographical pattern to cpDNA variation in *Eucalyptus* may be an important source of information on past plant distributions in Australia.

Key words: chloroplast DNA; *Eucalyptus*; hybridization; Myrtaceae; reticulate evolution.

Eucalyptus-dominated ecosystems support most of Australia's terrestrial biodiversity (Norton, 1997). Hence, understanding eucalypt evolution is fundamental to understanding the evolution and ecology of a large component of the Australian biota. *Eucalyptus* is a complex genus of ancient origin (Ladiges, 1997). While many taxa appear to be relictual, there is indication of recent speciation in certain groups (Pryor and Johnson, 1981; Prober, Bell, and Moran, 1990). Many species form complexes where extensive clinal variation between recognized taxa is common (Pryor and Johnson, 1971; Potts and Wiltshire, 1997) and reproductive barriers are weak (Potts and Wiltshire, 1997). Whether this intergradation is a result of recent primary differentiation or due to secondary intergradation through hybridization is debatable (Pryor and Johnson, 1981; Ladiges, 1997). If hybridization (reticulate evolution) was a primary factor, then phylogenetic reconstruction may be questionable (McDade, 1992; Rieseberg, 1995), and this would challenge the foundation of current taxonomy. Chloroplast DNA variation has been useful for elucidating taxonomic relationships at higher taxonomic levels in the eucalypts (Sale et al., 1993, 1996a; Ladiges, Udovicic, and Drinnan, 1995). In eucalypts, cpDNA is maternally inherited (Byrne, Moran, and Tibbits, 1993). However, at lower taxonomic levels, a recent study of cpDNA in the subgenus *Symphyomyrtus* has shown marked discordance between cpDNA and species phylogeny (Steane et al., 1998), suggestive of reticulate evolution.

The present study examines the utility of cpDNA in phylogenetic reconstruction in a group of closely related species in the subgenus *Monocalyptus*. Subgenus *Monocalyptus* contains more than 140 species that have been organized into one (Pryor and Johnson, 1971) or two (Johnson, 1976) sections, although at the morphological level, there are few obvious synapomorphic characters defining subgroups and no support for the two sections of Johnson (Ladiges, 1997). This is clearly exemplified by instability in the taxonomic treatment of *Monocalyptus* species on the island of Tasmania (Table 1). Tasmania is a large island southeast of mainland Australia, but was linked by land bridges during Quaternary glacial epochs (Marginson and Ladiges, 1982). Its flora displays a high level of endemism and relictual species (Kirkpatrick and Brown, 1984), including eucalypts (Ladiges, Humphries, and Brooker, 1983; Williams and Potts, 1996). The six Tasmanian endemic species of subgenus *Monocalyptus* have been the subject of numerous evolutionary studies [reviewed in Williams and Potts (1996); Potts and Wiltshire (1997)]. These species belong to the series *Amygdalinae* (Ladiges, Newnham, and Humphries, 1989; Table 1), are often morphologically highly differentiated (Sale et al., 1996b), but intergrade or hybridize in virtually all possible combinations (Williams and Potts, 1996). The dynamics of hybridization between two of the most morphologically differentiated of these taxa, *E. risdonii* and *E. amygdalina*, has been extensively studied using morphological (Potts and Reid, 1988; Whitham, Morrow, and Potts, 1994) and RAPD (Sale et al., 1996b) variation. The present study therefore aims to determine the level

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TABLE 1. Comparative published classifications of *Eucalyptus* subgenus *Monocalyptus* and outgroups sampled for cpDNA haplotype.

	Series, [subseries] and (superspecies)			
Subgenus and species	Ladiges, Humphries, and Brooker (1983), unless otherwise specified	Pryor and Johnson (1971)	Chippendale (1988)	Distribution in Australia[a]
Monocalyptus				
E. risdonii	Amygdalinae [Cocciferinae] (Risdonii)	Piperitae [Amygdalininae] (Risdonii)	Radiatae	Tas
E. tenuiramis	Amygdalinae [Cocciferinae] (Risdonii)	Piperitae [Amygdalininae] (Risdonii)	Radiatae	Tas
E. coccifera	Amygdalinae [Cocciferinae]	Piperitae [Amygdalininae]	Radiatae	Tas
E. nitida	Amygdalinae [Cocciferinae]	Piperitae [Amygdalininae] (Amygdalina)	Radiatae	Tas
E. amygdalina	Amygdalinae [Amygdalininae]	Piperitae [Amygdalininae] (Amygdalina)	Radiatae	Tas
E. pulchella	Amygdalinae [Pulchellinae]	Piperitae [Amygdalininae]	Radiatae	Tas
E. radiata subsp. *radiata*	Amygdalinae [Radiatinae] (Radiata)	Piperitae [Amygdalininae] (Amygdalina)	Radiatae	NSW, ACT, Vic
E. robertsonii[b]	Amygdalinae [Radiatinae] (Radiata)	Piperitae [Amygdalininae] (Amygdalina)	Radiatae	NSW, ACT, Vic
E. elata	Amygdalinae [Radiatinae] (Radiata)	Piperitae [Amygdalininae]	Radiatae	NSW, Vic
E. croajingolensis[c]	NA	NA	NA	SE NSW, E Vic
E. willisii subsp. *willisii*	Amygdalinae [Radiatinae] (Dives)	NA	Radiatae	SA to Vic
E. willisii subsp. *falciformis*	Amygdalinae [Radiatinae] (Dives)	NA	Radiatae	Grampians, Vic
E. dives	Amygdalinae [Radiatinae] (Dives)	Piperitae [Amygdalininae]	Radiatae	NSW, ACT, Vic
E. umbra	NA	Acmenioideae [−] (Acmenioides)	White-mahoganies	NSW, Qld
E. piperita subsp. *urceolaris*	Obliquae [Piperitanae]; Piperitinae [Piperititae][d]	Piperitae [Piperitinae]	Piperitales	NSW
E. obliqua	Eucalyptus[e]	Obliquae [Obliquinae]	Eucalyptus	Qld, NSW, Vic, Tas, SA
E. delegatensis	Fraxininae[d]	Obliquae [Delegatensinae]	Eucalyptus	NSW, ACT, Vic, Tas
Symphyomyrtus				
E. globulus	NA	Viminales [Globulinae]	Viminales	NSW, Vic, Tas
E. lansdowneana	NA	Odoratae [Odoratinae]	Porantheroideae	SA
Eudesmia				
E. ceracea	NA	NA	Miniatae	WA

[a] Abbreviations used: NA, not applicable; Tas, Tasmania; NSW, New South Wales; ACT, Australian Capital Territory; Vic, Victoria; SA, South Australia; Qld, Queensland; WA, Western Australia.

[b] *E. robertsonii* was raised to species status by Johnson and Hill (1990) from *E. radiata* ssp. *robertsonii*.

[c] *E. croajingolensis* was described by Johnson and Hill (1990). It is related to *E. radiata*.

[d] Ladiges, Prober, and Nelson, 1992.

[e] Ladiges, Newnham, and Humphries, 1989.

TABLE 2. Details of specimens collected for analysis of cpDNA haplotype in *Eucalyptus* subgenus *Monocalyptus* and outgroups.

Eucalyptus species and identifier	Collector	Natural locality	Geographic location[a]	Lat. (S)	Long. (E)	Herbarium specimen location and code[a]
E. risdonii 1	B. Potts	Risdon	SE Tas	42°50′	147°20′	TU: #RP264
E. risdonii 2	B. Potts	Meehan Range	SE Tas	42°51′	147°25′	TU: #MRTH C1
E. tenuiramis 1	B. Potts	Bicheno	SE Tas	41°53′	148°17′	TU: #BICH4
E. tenuiramis 2	B. Potts	Huon Road	SE Tas	42°54′	147°17′	TU: #HRC4
E. coccifera 1	B. Potts	Pine Lake	Cent. Tas	41°44′	146°42′	TU: #PL16
E. coccifera 2	R. Vaillancourt	Mt Wellington	SE Tas	42°53′	147°13′	TU: #GEM7
E. nitida 1	B. Potts	Port Davey	SW Tas	43°20′	145°53′	TU: #PD4
E. nitida 2	B. Potts	Flinders Island	NE Tas	~40°	~148°	TU: #N42
E. amygdalina 1	B. Potts	Risdon	SE Tas	42°50′	147°20′	TU: #AP242
E. amygdalina 2	B. Potts	Kingston	SE Tas	42°59′	147°18′	TU: #635
E. pulchella 1	B. Potts	Mt Nelson	SE Tas	42°54′	147°19′	TU: #633
E. pulchella 2	B. Potts	Harris Creek	SE Tas	43°15′	147°08′	TU: #68
E. radiata subsp. *radiata*	K. Rule	Upper Ferntree Gully	S Vic	37°54′	145°20′	TU: #GEM6
E. robertsonii	M. I. H. Brooker	Brindabella Range	SE NSW	35°27′	148°46′	TU: #12693
E. elata	K. Rule	Cann River	SE Vic	37°38′	149°09′	TU: #GEM5
E. croajingolensis	D. Nicolle	Holey Plains, Gippsland	SE Vic	38°14′	146°54′	TU: #29/16
E. willisii subsp. *willisii*	D. Nicolle	Holey Plains, Gippsland	SE Vic	38°14′	146°54′	TU: #32/13
E. willisii subsp. *falciformis*	D. Nicolle/K. Rule	Pomonal, Grampians	SW Vic	37°25′	142°20′	TU: #30/30
E. dives	K. Rule	Gembrook	S Vic	37°57′	145°33′	TU: #GEM4
E. umbra	B. Potts	Not recorded[b]	NSW or Qld			TU: #1537
E. piperita subsp. *urceolaris*	D. Nicolle	Nowra	SE NSW	35°06′	150°16′	TU: #51/30
E. obliqua	B. Potts	Mt Nelson	SE Tas	42°55′	147°18′	TU: #634
E. delegatensis	B. Potts	Mt Wellington	SE Tas	42°54′	147°14′	TU: #636
Outgroups						
E. globulus 1	G. McKinnon	Mt Nelson	SE Tas	42°56′	147°18′	TU: #637
E. globulus 2	D. Steane	Otway Ranges	Vic	38°40′	143°44′	TU: #DASteane 99
E. lansdowneana[b]	M. Sale	Gawler Ranges	SA	~32°	~136°	NA
E. ceracea[c]	M. Sale	King George Falls	WA	~14°	~127°	NA

[a] Abbreviations used: TU = School of Plant Science, University of Tasmania. Other abbreviations are as in Table 1.
[b] Sample obtained from Waite Arboretum, Adelaide, South Australia; appears to deviate from *E. umbra* slightly in the direction of a closely related form, *E. acmenoides* (I. Brooker, personal communication, CSIRO Plant Industry).
[c] Sample obtained from Kings Park, Perth, Western Australia.

and pattern of variation in cpDNA within this group of endemic species and place it in a broader phylogenetic framework.

MATERIALS AND METHODS

Plant material—Leaf material from 27 accessions representing 16 species of *Eucalyptus* subgenus *Monocalyptus* and three species from other subgenera to be used as potential outgroups (Table 2) was collected from plantations or native stands. Steane et al. (1998) showed that within subgenus *Symphyomyrtus* cpDNA can transgress species boundaries. Therefore, to be cautious, we choose outgroup taxa from other subgenera that were unlikely to share cpDNA with species of *Monocalyptus*. Specimens of *E. globulus*, 1 and 2, were identical to those used by Steane et al. (1998), in which they were designated GG1 and GG2, respectively. Specimens of *E. lansdowneana* and *E. ceracea* were identical to those used by Sale et al. (1996a), designated 1835B.K13 and 361/89, respectively. Care was taken to select individuals that were true to type.

DNA isolation and southern hybridization—Total cellular DNA was extracted from leaf tissue using a protocol based on the method of Wagner et al. (1987). Tissue (10 g) was ground to powder under liquid nitrogen using a mortar and pestle, then added to 50 mL extraction buffer (3.2% sorbitol, 5.0% polyethylene glycol 600, 0.05% bovine serum albumin, 0.05% spermine, 0.05% spermidine, 4% polyvinyl pyrrolidone 40, 0.05% 2-mercaptoethanol, 15 mmol/L EDTA, 50 mmol/L Tris, pH 8.0), on ice. The homogenate was filtered once through muslin, centrifuged (2000 *g*, 5 min), and the pellet was retained and suspended in 4 mL wash buffer (6.4% sorbitol, 0.1% 2-mercaptoethanol, 25 mmol/L EDTA, 50 mmol/L Tris, pH 8.0). The following reagents were added in order, with mixing: 1 mL of 5 mol/L NaCl, 0.8 mL of 8.6% hexadecyltrimethylammonium bromide/0.7 mol/L NaCl, and 1.6 mL of 5% N-laurylsarcosine. Samples were incubated at ambient temperature for 15 min, then at 55°C for 15 min, following which they were extracted twice with 8 mL chloroform : isoamyl alcohol (24:1), with mixing times of 30 min and 5 min, respectively, for the first and second extractions. The aqueous phase was separated from the organic phase by centrifugation (2000 *g*, 8 min). DNA was precipitated by addition of 6 mL ice-cold isopropanol and collected by centrifugation (2000 *g*, 5 min). The pellet was washed in 1 mL of 50% isopropanol/0.3 mol/L ammonium acetate for 30 min, air dried, resuspended in TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 7.4) containing RNase (10 μg/mL) and incubated at 37°C for 1 h. DNA was then reprecipitated with ethanol and 0.22 mol/L ammonium acetate, collected by spooling, washed in 70% ethanol, and stored at −20°C in TE buffer, pH 7.4.

DNA from each accession (1.5 μg per reaction) was digested with each of the following 19 restriction enzymes according to the manufacturers' instructions: *Alu* I, *Ase* I, *Bam*H I, *Ban* II, *Bcl* I, *Bgl* II, *Bst*N I, *Dde* I, *Dra* I, *Eco*R I, *Eco*R V, *Eco*0109 I, *Hin* d III, *Hin* f I, *Nco* I, *Nsi* I, *Ssp* I, *Xho* I, and *Xmn* I. Digested DNA was size fractionated by electrophoresis in 1.2% agarose for ~360 Vh, then transferred to nylon membrane by Southern blotting and cross-linking by exposure to UV radiation. Restriction fragments of phage lambda DNA cut with *Hin* d III were included as size markers. Nine chloroplast probes from *Petunia* (P1, P3, P4, P6, P10, P12, P14, P16, P20; Sytsma and Gottlieb, 1986) and one from *Nicotiana tabacum* (pTBa1; Shinozaki et al., 1986) were used. Probe DNA was labelled with ^{32}P using random primers (T7 QuickPrime kit, AMRAD Pharmacia Biotech, Australia). Following prehybridization for up to 24 h in 1 L of hybridization solution (0.5% nonfat dried milk powder, 1% SDS, 0.6 mol/L NaCl, 0.06 mol/L tri-

TABLE 3. Restriction site and size mutations scored for analysis of cpDNA haplotype in *Eucalyptus* subgenus *Monocalyptus* and outgroups.

Character No.	Character Code	Restriction enzyme/s and probe/s	Character details (fragment sizes in kb)[a]
1	a1	*Ase* I, P6	3.3 → 2.1 + x
2	a2	*Ban* II, P10	17.0 → 13.6 + 3.4
3	a3	*Bcl* I, P10	4.10 → 2.33 + 1.88
4	a4	*Eco*R V, P16	1.6 → 1.4 + x
5	a8	*Bgl* II, P3	9.55 → 7.40 + 2.15
6	a9	*Bgl* II, *Bam*H I, *Dra* I, P10	0.12 kb deletion
7	a11	*Xmn* I, P16	3.10 → 1.78 + 1.23
8	a12	*Dra* I, P16	2.9 → 1.9 + 1.0
9	a13	*Bam*H I, P6	8.6 → 4.8 + 3.6
10	a15	*Bam*H I, P14	2.70 → 1.38 + 1.13
11	b1	*Hin*f I, P14	0.63 → 0.54 + x
12	b6	*Dde* I, P4	(1.3) → 1.0 + x
13	b7	*Dde* I, P16	1.15 → 0.88 + x
14	b9	*Nsi* I, P10	3.60 → 2.85 + 0.75
15	c1	*Nsi* I, P4	2.5 → 1.6 + x
16	c2	*Nsi* I, P4	(6.7) → 3.6 + 3.1
17	c3	*Nsi* I, *Bst*N I, *Ase* I, P4	0.45 kb deletion
18	c4	*Dde* I, *Bcl* I, *Xmn* I, pTBal	0.20 kb deletion
19	c5	*Eco*R I, P1, P4	1.80 → 1.55 + x
20	c7	*Ase* I, P3	3.05 → 2.60 + x
21	c9	*Ase* I, P12, P20	3.78 → 1.55 + (2.23)
22	c11	*Ase* I, P4	(2.54) → 0.59 + 1.95
23	c12	*Ase* I, P20, pTBa1	2.2 → 1.6 + 0.60
24	c13	*Ssp* I, P10	2.77 → 1.78 + 0.88
25	c14	*Ssp* I, P10	2.95 → 2.77 + x
26	d1	*Ssp* I, pTBal	2.20 → 0.78 + (1.42)
27	d3	*Bgl* II, P6	7.8 → 7.6 + x
28	d4	*Bgl* II, P1, P4	16.8 → 12.5 + 4.3
29	d5	*Bgl* II, pTBal	5.8 → 4.3 + 1.5
30	d6	*Bgl* II, pTBal	2.8 → 2.7 + x
31	d8	*Ban* II, P14, P4	5.7 → 4.2 + x
32	d10	*Eco* RV, P10	5.00 → 3.65 + 1.35
33	d11	*Eco* RV, P10	2.95 → (2.40) + 0.55
34	d12	*Eco* RV, P10	5.80 → 2.40 + 2.95
35	d13	*Eco* 0109I, P6	7.9 → 4.8 + 3.1
36	d14	*Nco* I, P20	5.0 → 4.1 + x
37	d15	*Nco* I, P20	4.1 → 3.7 + x
38	ds18	*Ban* II, pTBal	10.0 → 8.5 + 1.5
39	ds20	*Dra* I, P10	2.1 → 1.1 + 1.0
40	e1	*Nco* I, P14	8.2 → 7.2 + 0.9
41	e2	*Dde* I, P3	3.2 → 2.7 + x
42	e3	*Dde* I, P6	1.90 → 1.08 + 0.82
43	e5	*Xmn* I, P20	5.70 → 3.20 + 2.50
44	e6	*Xmn* I, pTBal	2.90 → 2.60 + x
45	e7	*Bam*H I, P10	3.50 → 1.80 + 1.50
46	e8	*Bam*H I, P10	3.40 → 2.20 + 1.20
47	e9	*Hin*d III, P3	4.8 → (4.6) + x
48	e10	*Hin*d III, P20, P12	3.6 → 3.3 + x
49	e11	*Hin*d III, pTBal	4.95 → 3.80 + 1.15
50	e12	*Hin*d III, pTBal	4.33 → 2.75 + 1.58
51	f2	*Ase* I, P12, P20	2.23 → 1.75 + x
52	f3	*Ase* I, P4	2.60 → 1.85 + x
53	f6	*Bcl* I, P3	4.8 → 2.6 + (2.2)
54	f7	*Bam*H I, P4	7.4 → 1.1 + (6.3)
55	f8	*Bam*H I, P4	(6.3) → 4.9 + (1.4)
56	f9	*Bgl* II, P1, P4	12.5 → 9.5 + 2.5
57	f10	*Eco* 0109I, P1, P4	6.5 → 4.9 + 1.6
58	f11	*Ssp* I, P12	3.5 → 1.7 + 1.8
59	f14	*Bst*N I, P10	2.45 → 2.20 + x
60	g1	*Eco* 0109I, P3, P6	10.0 → 6.0 + 4.0
61	g3	*Dde* I, pTBal	2.6 → 1.3 + (1.3)
62	g4	*Dde* I, pTBal	2.20 → 2.05 + x
63	g8	*Nco* I, P6	7.3 → 4.9 + 1.8
64	g9	*Nco* I, P6	7.3 → 5.9 + 1.4
65	hj15	*Ase* I, P3	1.60 → 1.33 + x
66	hj21	*Xmn* I, P3	1.39 → 1.27 + x
67	hj22	*Xmn* I, P3	8.4 → 6.5 + 1.7
68	hj23	*Xmn* I, P3	3.70 → 3.28 + x
69	hj25	*Xmn* I, P20, P12	8.20 → 4.60 + 3.60
70	hj47	*Eco* 0109I, P4	2.6 → 1.3 + (1.3)
71	hj52	*Dra* I, P10	2.30 → 2.09 + x
72	hj88	*Eco* 0109I, P6, P10	24.0 → 3.5 + 20.5
73	z1	*Eco*R V, pTBal	16.5 → 8.5 + 8.0
74	z2	*Ase* I, pTBal	9.0 → 6.8 + 2.2
75	z3	*Bcl* I, P3, P16	7.4 → 4.8 + 2.6
76	z4	*Xmn* I, P3	11.0 → 9.2 + 1.8
77	z5	*Xmn* I, P3	11.0 → 5.5 + (5.5)
78	z6	*Dra* I, P3	7.4 → 7.2 + x

[a] The actual measured sizes of restriction fragments are given. Missing restriction fragments are denoted as ''x''. Brackets around a number indicate that a fragment of this size was deduced but could not be unequivocally detected due to a coinciding fragment of the same size.

sodium citrate, pH 7.0), all blots were probed simultaneously for at least 18 h at 65°C in ~300 mL of hybridization solution containing 400 ng cpDNA probe and 5 ng lambda probe. Blots were washed of excess probe with at least 750 mL of wash buffer (0.5% SDS, 0.3 mol/L NaCl, 0.03 mol/L trisodium citrate, pH 7.0) until background radiation dropped, and exposed to X-ray film (X-OMAT AR, Eastman Kodak Co., New York) at −80°C for up to 76 h with intensifying screens (Hyperscreen, Amersham, UK).

Data analysis—Autoradiographs were scored for presence or absence of restriction sites and for size mutations (where three or more enzymes indicated a conserved alteration in fragment size with a single probe). A data matrix was constructed comprising 78 characters across the 27 *Eucalyptus* accessions (Appendix). One percent of the matrix cells was scored as missing data. Where individuals had identical haplotypes, these were represented by a single individual to simplify cladistic analysis. Cladograms were generated by the parsimony software package PAUP version 3.1.1 (Swofford, 1993) using Wagner parsimony. The condensed data matrix was analyzed using the exact branch-and-bound search option, with ''Furthest'' addition sequence, tree bisection-reconnection (TBR) branch swapping, and the save all minimal tree option (MULPARS) on. Bootstrap analysis was carried out using a heuristic search option, 1000 bootstrap replicates, TBR swapping, and 100 replicates of random addition sequence within each bootstrap replicate. Trees were rooted using *E. ceracea* from subgenus *Eudesmia* as the designated outgroup; replacing *E. ceracea* with various combinations of *E. globulus, E. lansdowneana* (both from subgenus *Symphyomyrtus*), and *E. ceracea* did not change the topology of the in-group (*Monocalyptus*). In addition, characters were mapped to the branches of an individual phylogenetic tree, enabling the identification of homoplasious characters.

RESULTS

Restriction digestion of total leaf DNA with 19 different restriction endonucleases, combined with hybridization to ten probes specific to different regions of the chloroplast genome, enabled scoring of 78 distinct characters (Table 3; Appendix), of which 42 were autapomorphic, giving 36 parsimony-informative characters. Three characters were deletions; the remainder were gains or losses of restriction sites. While all enzymes except *Alu* I and *Xho* I provided useful data, five enzymes were exceptionally useful in detecting cpDNA polymorphisms across the full range of *Eucalyptus* species analyzed: *Ase*

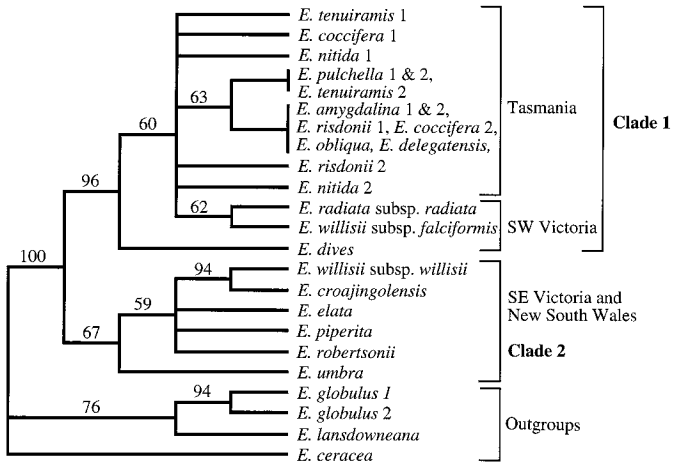

Fig. 1. Strict consensus of six equally parsimonious trees (length = 89; CI = 0.76, excluding autapomorphic characters) obtained from a branch-and-bound search of cpDNA haplotypes in *Eucalyptus*, with *E. ceracea* (subgenus *Eudesmia*) as designated outgroup. Bootstrap percentages are shown above the branches.

I, *Bam* H I, *Bgl* II, *Dde* I, and *Xmn* I. Within the monocalypts, the enzymes revealing most polymorphisms were *Ase* I, *Bam*H I, *Bgl* II, and *Dde* I. Of all cpDNA polymorphisms recorded, 77% involved the large single-copy region of the chloroplast genome or its boundary with the inverted repeat region.

Wagner parsimony analysis of the data matrix using the branch and bound algorithm generated six trees, each with 89 steps (including autapomorphies) and consistency index of 0.76 (excluding autapomorphic characters). The strict consensus cladogram (shown with bootstrap values in Fig. 1) indicated that the cpDNA haplotypes of *Monocalyptus* were monophyletic, but also divided into two major, well-supported clades based on geographical origin. While the observed distribution of polymorphism distinguished clearly between subgenera *Monocalyptus*, *Symphyomyrtus*, and *Eudesmia* (Fig. 2), the cpDNA haplotype was not species specific and even crossed series boundaries. Individual accessions of *E. amygdalina*, *E. coccifera*, and *E. risdonii*, representing series *Amygdalinae* from the Hobart region, shared a common cpDNA haplotype with accessions of *E. obliqua* (series *Eucalyptus*) and *E. delegatensis* (series *Fraxininae*) from the same area, while a duplicate accession of *E. risdonii* also from the same area was distinguishable from this haplotype by four characters. All Tasmanian monocalypts fell within a large clade (clade 1) also containing monocalypts from southwestern Victoria, while a separate clade (clade 2) contained monocalypts of southeastern Victoria and southeastern New South Wales (Fig. 3). Within these loose geographical groupings, further foci of closely related haplotypes occurred, most notably the strongly supported linkage of accessions of *E. willisii* subsp. *willisii* and *E. croajingolensis*, both from Holey Plains, Gippsland in Victoria.

Mapping of characters to the branches of an individual cladogram (Fig. 2) revealed a comparative lack of variation within the Tasmanian monocalypts, with most of the observed differences being due to autapomorphies or homoplasious characters. Monocalypts from the mainland

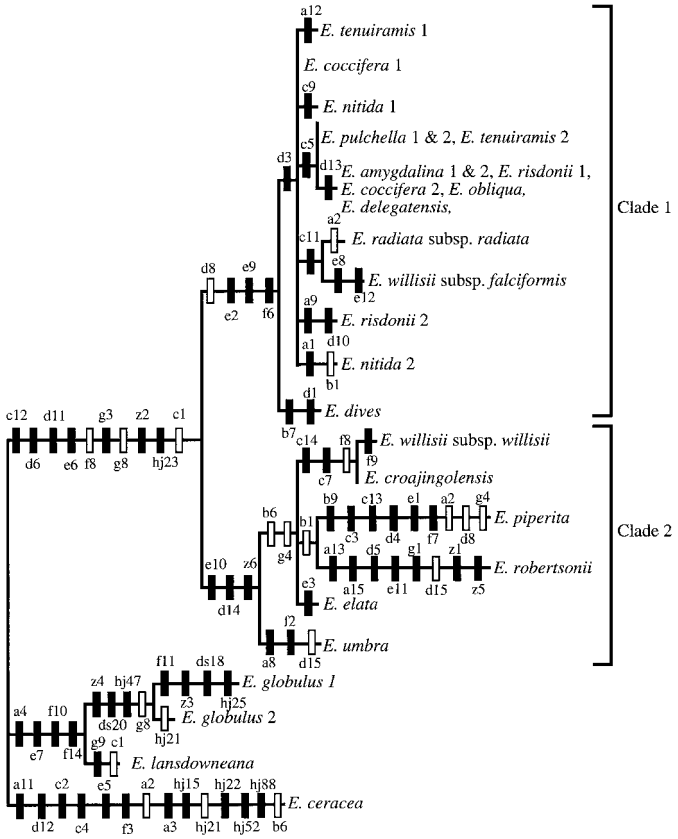

Fig. 2. One of six equally parsimonious trees obtained from a branch and bound search of the cpDNA data matrix, with *E. ceracea* (subgenus *Eudesmia*) as designated outgroup. Characters have been mapped to the branches they support. Open boxes denote homoplasious characters; black boxes denote nonhomoplasious characters (synapomorphic or autapomorphic).

of Australia showed much more variation in their haplotypes. The two monocalypt individuals originating from southeastern New South Wales, *E. piperita* subsp. *urceolaris* (series *Piperitinae* of Ladiges, Prober, and Nelson, 1992) and *E. robertsonii* (series *Amygdalinae*), both displayed a high degree of autapomorphy within this study. While single aberrations within the sampled individuals cannot be excluded, it seems more likely that these individuals represent monocalypt populations with distinctive cpDNA haplotypes.

DISCUSSION

The cpDNA of *Eucalyptus* subgenera *Monocalyptus* and *Symphyomyrtus* formed two monophyletic clades, supporting earlier molecular analyses that also demonstrated monophyly of these subgenera using cpDNA data from different accessions (Sale et al., 1993, 1996a). While some congruities between the cpDNA phylogeny and the morphologically based cladistic analysis of Ladiges, Humphries, and Brooker (1983) were found at lower taxonomic levels, overall the cpDNA phylogeny within *Monocalyptus* was more indicative of the geographic proximity of the accessions than of published taxonomic relationships between species based on morphology (Table 1). Regardless of series, nine of the ten ac-

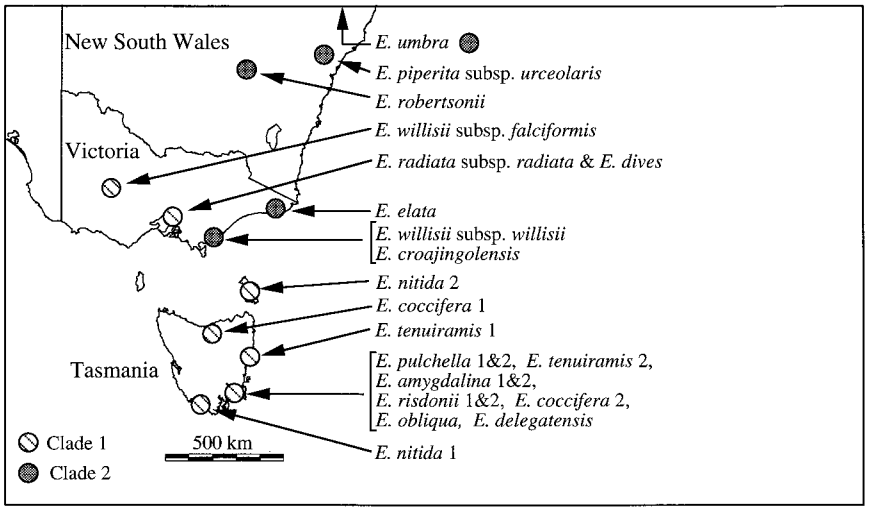

Fig. 3. Regional map showing geographical origin of sampled monocalypts within southeastern Australia and their distribution within the two major cpDNA clades described in Fig. 1.

cessions of *Monocalyptus* sampled close to Hobart in southeastern Tasmania formed a clade comprising only two haplotypes (clade supported by character c5) for a total of seven species. This clade fell within a larger clade (clade 1) containing the remaining accession from the Hobart region (*E. risdonii* 2), four accessions from elsewhere in Tasmania, and two accessions from southwestern to southern central Victoria on the Australian mainland. The separation of clade 1 from clade 2, which contained only accessions from southeastern Victoria and New South Wales, also transcended taxonomic groupings. Overall, five out of seven species in which duplicate trees were sampled were polyphyletic in their cpDNA. This conforms exactly to the general pattern of variation in cpDNA observed by Steane et al. (1998) in *Symphyomyrtus* series *Viminales*. There is clearly a high level of intraspecific variation in cpDNA in *Eucalyptus* species, and this is coupled with extensive sharing of related haplotypes by species in the same geographical area.

Little cpDNA variation was found in the Tasmanian *Amygdalinae* species compared to that found on the mainland of Australia. Recent divergence, whereby time since divergence is insufficient for numerous mutations to accumulate in the cpDNA, of some Tasmanian taxa may be part of the explanation. The Tasmanian *Amygdalinae* species are all endemic (Marginson and Ladiges, 1982) and, despite marked morphological differentiation, speciation may not yet have occurred (Wiltshire, Potts, and Reid, 1992). These results give little hope of finding species-specific cpDNA markers for the Tasmanian *Amygdalinae*. The Tasmanian *Monocalyptus* contained only cpDNA of clade 1, whereas mainland species had cpDNA of both clades 1 and 2. As *E. dives* from southern central Victoria appears to be basal to clade 1 (branch supported by one synapomorphic character), it is possible that the Tasmanian cpDNA haplotypes evolved from a common ancestral cpDNA found in that part of Victoria. This link may be indicative of the direction of colonization of Tasmania. The geographic separation of accessions with haplotypes from clades 1 and 2 and the lack of variation in the cpDNA of Tasmanian species may have been caused by a bottleneck event in Tasmania. This bottleneck could have occurred during a glacial event, when eucalypt forest cover was restricted in Tasmania (Kirkpatrick and Fowler, 1996). Alternatively, there could have been a biogeographical barrier (e.g., geology, climate) across Victoria that prevented cpDNA of clade 2 from moving into western Victoria and Tasmania.

Three hypotheses may be presented to explain the overall lack of congruence between the cpDNA and species phylogenies: (1) convergent evolution of cpDNA or morphological species; (2) lineage sorting of cpDNA; and (3) hybridization and introgression (Soltis et al., 1991; Steane et al., 1998). Convergent evolution and lineage sorting could have played a role in the discordance between the species and cpDNA phylogenies, especially since there is evidence for recent divergence between species. Relatively recent speciation could result in morphologically different species possessing undifferentiated haplotypes. Likewise, haplotypes that differ by only a few characters may converge more easily than those differing by more characters. Recognition sites for restriction enzymes comprise several base pairs. Gain or loss of a restriction site can be achieved via changes in one or more bases of the recognition site. Thus, even if a restriction site is present or absent in two organisms, the process leading to the presence or absence may be different (convergent evolution) resulting in false homology. However, the potential for convergence in cpDNA decreases sharply as the number of character differences increases, hence this is unlikely to account for similarities between series. The observed results cannot be accounted for completely by lineage sorting and convergent evolution. For example, there is more divergence in cpDNA within *E. risdonii* (a highly localized endemic) than between *E. risdonii* and individuals from two different series (*E. obliqua* and *E. delegatensis*). These results are best explained by hybridization followed by introgression, possibly following the stepping stone model of Soltis et al. (1991). Furthermore, numerous characters separate samples of the two subspecies of *E. willisii*, a finding that is difficult to explain via the mechanisms of lin-

eage sorting or convergent evolution of cpDNA, but that could be explained by introgression of cpDNA. However, the possibility that these subspecies may in fact be derived from two very divergent lineages that have converged in their adult morphology cannot be discounted. Certainly, the results of Newnham, Ladiges, and Whiffin (1986) show as much differentiation in seedling morphology and volatile leaf oils between the two subspecies of *E. willisii* as they do between the two taxa *E. willisii* and *E. pauciflora*, from series *Amygdalinae* and series *Psathroxyla*, respectively.

The possibility of introgression of cpDNA from one lineage to another is partially supported by morphological evidence of hybridization. Within series, many species of *Monocalyptus* have been observed to hybridize and in some cases form extensive intergrade zones (Williams and Potts, 1996; Wiltshire, Potts, and Reid, 1992). It is therefore to be expected that species of the same series could share the same chloroplast genome. Although recorded, natural hybrids between series are much less common, and intergrade zones have not been observed (Potts and Reid, 1983; Williams and Potts, 1996). The observation that species from different series may share a common haplotype is therefore surprising. These data suggest that hybridization may be considerably more extensive and more significant in *Eucalyptus* than suspected previously. Further sampling will be necessary to confirm this indication. Interspecific hybridization and introgression were also strongly implicated in a recent study of cpDNA of species from subgenus *Symphyomyrtus* series *Viminales* (Steane et al., 1998). These results led the authors to conclude that cpDNA may not be useful in phylogenetic reconstruction at low taxonomic levels within this subgenus. It is now possible to generalize this conclusion to *Eucalyptus* overall, since *Symphyomyrtus* and *Monocalyptus* together comprise the two most speciose subgenera within *Eucalyptus*. Nevertheless, as demonstrated in other plant species in Europe (Dumolin-Lapègue et al., 1997) and North America (Soltis et al., 1997), understanding the geographic pattern to cpDNA variation in *Eucalyptus* may be a useful source of information on past plant distributions in Australia.

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APPENDIX. Data matrix showing scoring of characters for cpDNA haplotype analysis in *Eucalyptus* subgenus *Monocalyptus* and outgroups. Characters are defined in Table 3. Where the character is a deletion (characters 6, 17, and 18), the score of 1 indicates the presence of the deletion. Where the character is a restriction site (remaining characters), the score of 1 indicates presence of the site, 0 indicates absence. A score of ? denotes missing data.

	Character number							
Specimen ID	10	20	30	40	50	60	70	
E. risdonii 1	0111100110	0000110010	1000111101	1101000101	1000001110	0011001101	0010000011	00101001
E. risdonii 2	0111110110	0000110000	1000111101	1001100101	1000001110	0011001101	0010000011	00101001
E. tenuiramis 1	0111100010	0000110000	1000111101	1101100101	1000001110	0011001101	0010000011	00101001
E. tenuiramis 2	0111100110	0000110010	1000111101	1101100101	1000001110	0011001101	0010000011	00101001
E. coccifera 1	0111100110	0000110000	1000111101	1101100101	1000001110	0011001101	0010000011	00101001
E. coccifera 2	0111100110	0000110010	1000111101	1101000101	1000001110	0011001101	0010000011	00101001
E. nitida 1	0111100110	0000110000	0000111101	1101100101	1000001110	0011001101	0010000011	00101001
E. nitida 2	1111100110	1000110000	1000111101	1101100101	1000001110	0011001101	0010000011	00101001
E. amygdalina 1	0111100110	00?0110010	1000111101	1101000101	1000001110	0011001101	0010000011	00101001
E. amygdalina 2	0111100110	0000110010	1000111101	1101000101	1000001110	0011001101	0010000011	00101001
E. pulchella 1	0111100110	0000110010	1000111101	1101100101	1000001110	0011001101	0010000011	00101001
E. pulchella 2	0111100110	0000110010	1000111101	1101100101	1000001110	0011001101	0010000011	00101001
E. radiata spp. *radiata*	0011100110	0000110000	1100111101	1101100101	1000001110	0011001101	0010000011	00101001
E. robertsonii	0111100101	1100110000	1000110111	01011?11?1	0000000?00	0001001100	0110000011	00001010
E. elata	0111100110	0100110000	1000110101	0101110101	0100000???	0001001101	0110000011	00?01000
E. croajingolensis	0111100110	0100110001	1000010101	0101110101	0000000010	0001101101	0110000011	00101000
E. willisii ssp. *willisii*	0111100110	0100110001	1000010101	0101110101	0000000010	0001111101	0110000011	00101000
E. willisii ssp. *falciformis*	0111100110	0000110000	1100111101	1101100101	1000011111	0011001101	0010000011	00101001
E. dives	0111100110	0010110000	1000100101	1101100101	1000001110	0011001101	0010000011	00101001
E. umbra	0111100110	0000110000	100011?101	01011?1101	000000??10	1001001101	0010000?11	00101?00
E. piperita ssp. *urceolaris*	0011100110	1101111000	1001110001	1101110100	000000?010	0000001101	0010000011	00101000
E. obliqua	0111100110	0000110010	1000111101	1101000101	1000001110	0011001101	0010000?11	00101?01
E. delegatensis	0111100110	0000110010	1000111101	1101000101	1000001110	0011001101	0010000011	00101001
E. globulus 1	0110100110	0000010000	1010110100	0111100011	??01100110	0001100011	??10000100	00110101
E. globulus 2	0110100110	0000010000	1010110100	0111100111	0001100110	0001100111	10?0010110	00111101
E. lansdowneana	0110100110	0000110000	1010110100	01111?0101	0001100?10	0001100?1	1001000111	0011?001
E. ceracea	0001101110	0100000100	1010110100	0110100101	0011000110	0101101101	1000111111	11111001